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Rapid Detection of the Common Mediterranean α-Globin Deletions/Rearrangements Using PCR

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The most frequent molecular lesions causing α-thalassemia are deletions of one or more α-globin genes. Detection of these deletions generally requires genomic Southern analysis, which is cumbersome and time consuming. We have designed new sets of primers for PCR identification of the common Mediterranean α-globin gene rearrangements, including the $-\alpha^{3.7}$ deletion and the $\alpha\alpha\alpha^{anti3.7}$ triplication, the $-\alpha^{4.2}$ deletion, and the $--^{Med}$ allele. We have established reaction conditions that provide easily interpretable, unambiguous diagnoses. Some of the PCR reactions are multiplex, simultaneously identifying several genotypes, thus reducing the time and cost of screening and prenatal testing. The use of these methods should facilitate carrier screening and identification of couples at risk for α-thalassemia. Am. J. Hematol. 58:306–310, 1998. © 1998 Wiley-Liss, Inc.

Key words: α-thalassemia; carrier screening; multiplex PCR; thalassemia intermedia; population screening

INTRODUCTION

The two α-globin genes are present within a 4-kb duplicated region leading to many rearrangements, including deletions and triplications [1,2]. These may have profound effects on concurrent β-thalassemia, either heterozygous [3–7] or homozygous [8–10]. Therefore, the molecular diagnosis of α-thalassemia is of great importance. A rapid methodology to detect α-globin rearrangements would greatly facilitate determining the double heterozygous state for α- and β-thalassemia. As such, it would allow more definitive evaluation of β-thalassemia carriership and identification of couples at risk. It would also facilitate epidemiologic studies to determine the frequency of these alleles. For these reasons we undertook to develop methods for rapid molecular diagnosis of α-thalassemia.

Previous methods to detect the common α-globin rearrangements in the Mediterranean population were based on genomic blotting using at least two restriction endonucleases (Bg1II, BamHI) with two different probes (α and ζ) [11,12]. The method is highly time consuming, and is, therefore, problematic for routine use in diagnosis and genetic counseling. The multiple repeat elements and the high GC-content of the α-globin locus impose severe limitations in designing suitable specific primers for PCR. The difficulty in developing satisfactory PCR methodologies is reflected in the large number of investigators that found it necessary to approach this problem [13–18].

We attempted to apply the published methods to molecular diagnosis of α-globin gene rearrangements, but we found them unsatisfactory for our purposes. The multiplex method of Bowden et al. [13], which detects the $--^{Med}/\alpha\alpha$, does not detect the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions, which are common in our region. The method described by Dode et al. [14] is designed to identify only the $-\alpha^{3.7}$ deletion and the corresponding triplication. Another limitation of this method is that it requires an additional step of pre-digestion of the DNA with BamHI. The multiplex method reported by Bowie et al. [16] requires an automated DNA sequencer, which is not available in many laboratories. Furthermore, this approach relies on amplification of intergenic sequences and may, thus, inadvertently provide misleading results. The strategy devised by Baysal and Huisman [15] to detect the $-\alpha^{3.7}$ deletion yields the same size PCR products for both the normal

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TABLE I. Sequence and Location of PCR Primers

Primer	Sequence	Gene Bank accession no.	Coordinates	
P51	CTGCACAGCTCCTAAGCCAC	J00153	7254–7273	α2
			11072–11091	α1
P52	CCTCCATTGTTGGCACATTCC	J00153	7531–7551[a]	α2
P54	CTCAAAGCACTCTAGGGTCCA	J00153	11497–1517	α1
P55	GTCCACCCCTTCCTTCCTCA	J00153	5687–5706	Y2
			9247–9266	Y1
P59	CTCTAGGTCACCCTGTCATCA	J00184	26–46	Ψζ1
P60	CTCTGTCGTGTAGACGCCGA	M33022	423–442	θ2
P71	TACCCATGTGGTGCCTCCATG	J00153	3068–3088	Ψα1
P72	TGTCTGCCACCCTCTTCTGAC	J00153	8894–8915	NH-II[b]

[a]A nearly identical sequence (20/21 nt) is present at coordinates 3280–3300, 3′ to ψα1.
[b]NH-II = nonhomologous region II.

and the deletion chromosomes, necessitating that the test be performed in two separate tubes, without an internal standard.

We have developed a new set of primers for the detection of the common Mediterranean α-globin gene rearrangements, including the $-\alpha^{3.7}$ deletion and $\alpha\alpha\alpha^{anti3.7}$ triplication, the $--^{Med}$ chromosomes and the $-\alpha^{4.2}$ deletion. The PCR reactions simultaneously identify several genotypes, thus reducing the time and cost of screening and prenatal testing.

MATERIALS AND METHODS

DNA was isolated from peripheral blood leukocytes according to standard procedures [12].

Table I presents the nucleotide sequences of the primers used in the different PCR reactions, and Figure 1 shows their locations within the cluster. PCR reaction A (see Table II) is a multiplex reaction containing the following primers: P51 (60 ng), P52 (40 ng), P54 (60 ng), P59 (40 ng), and P60 (40 ng). Reaction B contains primers P55 (60 ng) and P54 (60 ng). Reaction C is a multiplex reaction, which contains primers P71 (40 ng), P72 (60 ng), and P52 (20 ng). Reaction D contains primers P55 (60 ng) and P52 (60 ng).

All PCR reactions were performed with Taq DNA polymerase (MBI Fermentas) using ~1 μg of genomic DNA. Each 50-μl reaction contained the manufacturer's buffer, 1 mM $MgCl_2$, 2.5 μg BSA, and 10% DMSO. Hot start PCR was used. The program was initiated with denaturation at 94°C for 5 min followed by 86°C for 2 min, during which the enzyme was added. Thereafter, denaturation was for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min (for PCR reaction A) or 2 min (for PCR reactions B, C, and D), at 72°C, with an additional 10 min at 72°C in the last cycle for all reactions. Thirty-five cycles were usually sufficient. The PCR products of reaction A were analyzed on 2% NuSieve gels in TBE, for reactions B and D on 1.0% agarose gels in TAE, and for reactions C on 1.4% agarose gels in TBE.

RESULTS

Table II presents the different PCR fragments that are expected for each allele with each primer pair. It can be seen that the different alleles can be diagnosed unambiguously with one or more primer pairs.

Multiplex PCR A was designed to specifically detect the presence of either α-globin gene, α2, and/or α1, as well as the $--^{Med}$ allele. The two α genes are selectively amplified by 3 primers: a common 5′ primer from the intron 2/exon 3 junction (P51, Fig. 1A) and two unique 3′ primers derived from the 3′ untranslated regions of α2 (P52) and α1 (P54) (Fig. 1A). Primers P59 and P60, which span the breakpoint junction [19], amplify the $--^{Med}$ allele (Fig. 1A). The amplification products are distinguishable in size (Table II), allowing multiplex analysis. Furthermore, the amplification of the α2 and/or α-globin genes serves as an internal positive control for the PCR reaction. Thus this multiplex reaction identifies 3 genotypes (Fig. 2A): $--^{Med}/\alpha\alpha$ (lane 1), $-\alpha/-\alpha$ (lane 3), and $--^{Med}/-\alpha$ (lane 4). Lane 2 could represent either αα/αα or $-\alpha/\alpha\alpha$. The differentiation between these genotypes using PCR reaction A necessitates quantification of the PCR products. This not only requires an additional step, but can also lead to ambiguous results if the PCR reaction is not within the linear range.

We have, therefore, designed additional PCR reactions that detect the specific single gene deletions $-\alpha^{3.7}$ and $-\alpha^{4.2}$. PCR reaction B (P55–P54) identifies heterozygotes for the $-\alpha^{3.7}$ deletion. P55 is derived from the Y-boxes (Fig. 1B), and P54 from the unique sequence at the 3′ untranslated region of α1. A normal α1 gene yields a 2,271-bp PCR product (Y_1IIIZ_1) while the $-\alpha^{3.7}$ deletion allele yields a 2,013-bp fragment (Y_2Z_{2-1}) (Fig. 1B). The following genotypes can be distinguished (Fig. 2B): αα/αα (lane 5), 2,271 bp; $-\alpha^{3.7}/-\alpha^{3.7}$ (Fig. 2B, lane 6), 2,013

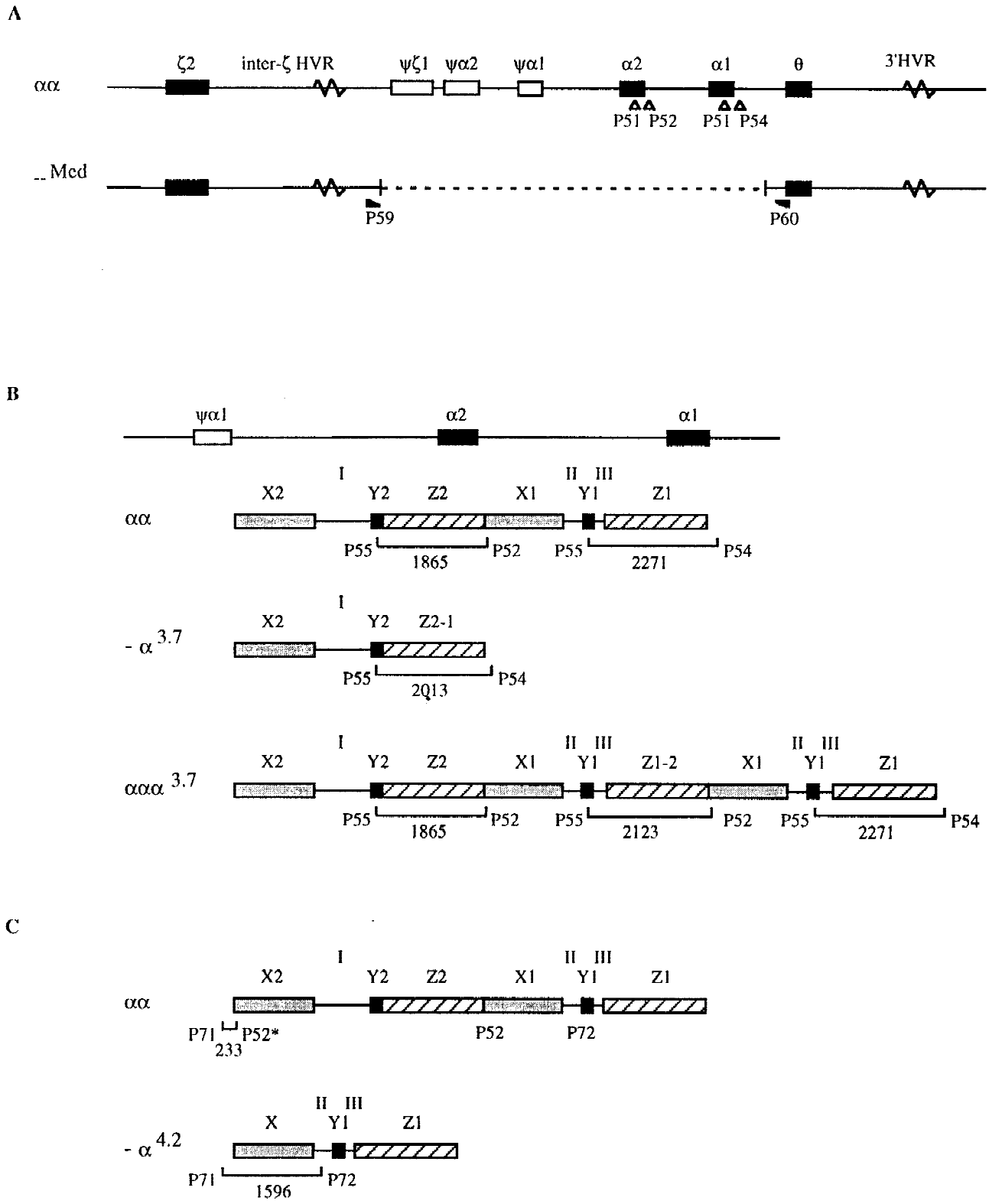

Fig. 1. A: Physical maps of the human α-globin cluster. Solid boxes represent functional genes and open boxes represent pseudogenes. The dashed line designates the --^{Med} deletion. The location of the PCR primers is shown below the maps. B, C: The arrangement of the homologous (X, *Y*, and Z boxes, denoted as stippled, black, and hatched, respectively) and non-homologous regions (I, II, and II) in the α-globin cluster is shown, in addition to the products of unequal crossing over between the Z boxes (-$\alpha^{3.7}$ and $\alpha\alpha\alpha^{anti3.7}$) and X boxes (-$\alpha^{4.2}$). The locations of the PCR primers and the sizes of the expected fragments are shown.

TABLE II. Expected PCR Product Sizes (Base Pairs) Using Various Primer Combinations*

	PCR reaction						
	A			B	C		D
Alleles	P59-P60	P51-P52	P51-P54	P55-P54	P71-P72	P71-P52	P55-P52
αα	—	298	446	2,271	—	233	1,865
$--^{Med}$	561	—	—	—	—	—	—
$-\alpha^{4.2}$	—	—	446	2,271	1,596	NT	—
$-\alpha^{3.7}$	—	—	446	2,013	—	233	—
$\alpha\alpha\alpha^{anti3.7}$	—	298	446	2,271	—	233	1,865; 2,123

*NT = not tested, as we did not have access to DNA of a homozygote. P = primer.

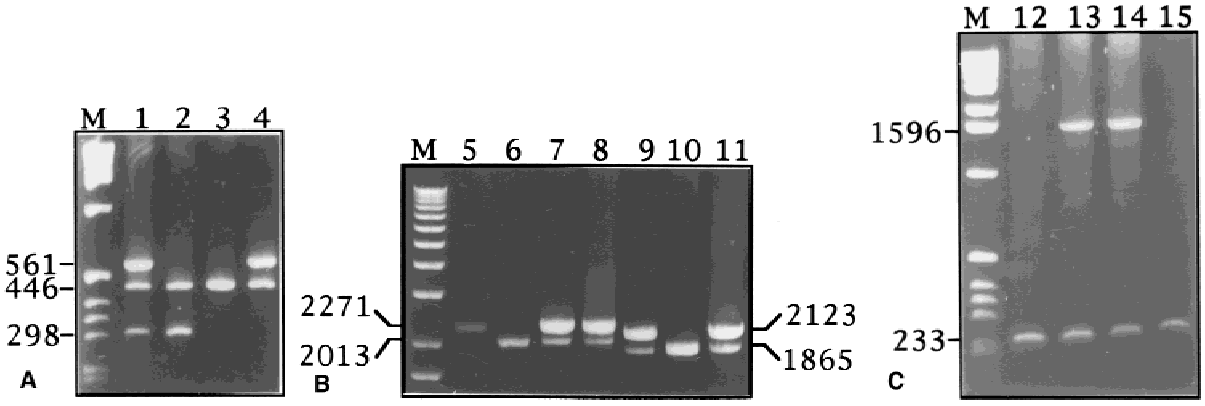

Fig. 2. Analysis of the PCR products. A: Multiplex reaction A. M, 1 kb Marker; lane 1, $--^{Med}/\alpha\alpha$; lane 2, normal αα/αα; lane 3, -α/-α; lane 4, $--^{Med}/-\alpha$. B: Detection of $-\alpha^{3.7}$ deletion or $\alpha\alpha\alpha^{anti3.7}$ triplication. Lanes 5–8 present products of PCR reaction B. M, 1 kb marker; lane 5, normal αα/αα; lane 6, $-\alpha^{3.7}/-\alpha^{3.7}$; lane 7, $-\alpha^{3.7}/\alpha\alpha$; lane 8, $-\alpha^{3.7}/-\alpha^{4.2}$. Lanes 9–11 show PCR reaction D. Lane 9, $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha$ triplication; lane 10, normal αα/αα; lane 11, $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha\alpha^{anti3.7}$. C: Detection of the $-\alpha^{4.2}$ deletion by multiplex reaction C. M, 1 kb marker; lane 12, normal αα/αα; lane 13, $-\alpha^{3.7}/-\alpha^{4.2}$; lane 14, $-\alpha^{4.2}/\alpha\alpha$; lane 15, $-\alpha^{3.7}/-\alpha^{3.7}$.

bp; and $-\alpha^{3.7}/\alpha\alpha$ (Fig. 2B, lane 7), 2,271 and 2,103 bp. The genotype $-\alpha^{3.7}/-\alpha^{4.2}$ (Fig. 2B, lane 8) is indistinguishable from the $-\alpha^{3.7}/\alpha\alpha$, since the $-\alpha^{4.2}$ chromosome yields the same size fragment as the normal chromosome. However, individuals who were diagnosed as either αα/αα or -α/αα by multiplex reaction A (Fig. 2A, lane 2) and who show the 2,103 bp diagnostic of the $-\alpha^{3.7}$ deletion, must have $-\alpha^{3.7}/\alpha\alpha$ genotype. To distinguish between $-\alpha^{4.2}/\alpha\alpha$ and αα/αα, both of which are identical in reactions A (Fig. 2A, lane 2) and B (Fig. 2B, lane 5), we perform PCR reaction C, which gives a positive identification of the $-\alpha^{4.2}$ allele (1,596-bp fragment). Compound heterozygotes for $-\alpha^{3.7}/-\alpha^{4.2}$, who yield a 446-bp band in multiplex reaction A, are diagnosed by the presence of the 2,103-bp band in reaction B, and the 1,596-bp fragment in reaction C.

The precise deletion junction of the $-\alpha^{4.2}$ deletion has not been determined [20], creating a problem in designing appropriate primers. Primers P71 and P72 identify unique sequences: primer P71 is derived from ψα1, and P72 from non-homologous region II (Fig. 1C). To provide an internal control, we perform PCR reaction C with an additional primer, P52. This primer, which is derived from the 3′ untranslated region of α2, anneals to a nearly identical (20/21 nt) sequence 3′ to ψα1 (Fig. 1C, denoted P52*), yielding a 233-bp fragment (Fig. 2C).

PCR reaction D detects the $\alpha\alpha\alpha^{anti3.7}$ triplication, which by itself has no hematological phenotype. However, in combination with heterozygous β-thalassemia, it causes a mild to moderate clinical picture of thalassemia intermedia. The PCR reaction is performed with primers P55 (derived from the Y-boxes) and P52 (Fig. 1B), from the unique region of α2. The triplication allele yields two bands: a 2,123-bp band and the normal 1,865-bp band (Fig. 2B, lanes 9–11). This PCR reaction provides a quick screening tool for the presence of $\alpha\alpha\alpha^{anti3.7}$ triplication. Differentiation between homo- or heterozygosity for the triplication necessitates genomic analysis.

Under the conditions described above, nonspecific amplification products were not seen in any of these 4 PCR reactions. We found that the addition of DMSO and BSA was absolutely essential. Without either one of these reagents, the reaction did not work or nonspecific bands were obtained despite the high annealing temperatures.

DISCUSSION

The PCR reactions described here can be used for rapid screening and detection of 3 different deletions, triplication (a total of 4 mutant alleles and the normal allele), and combinations thereof, in individuals suspected of carrying α-thalassemia. The first PCR reaction facilitates the identification of 3 different genotypes in a single PCR tube, and identifies Hb H disease that is caused by deletion of 3 genes. Furthermore, it distinguishes between carriership of two genes deleted in cis ($--^{Med}$) or in trans (-α/-α), which is of great importance for carrier screening and genetic counseling. The second PCR reaction identifies the $-\alpha^{3.7}$ chromosome and differentiates between homozygosity and heterozygosity for this allele. The $-\alpha^{4.2}$ chromosome is identified using the third PCR reaction. For most individuals suspected of carrying an α-globin gene deletion or rearrangement, two PCR reactions enable unambiguous identification of the complete genotype.

For the past months, our laboratory has been performing these PCR analyses in parallel with genomic blotting and there have been no discrepancies in results. We therefore anticipate that these PCR strategies should prove to be valuable for the purposes of screening carriers or populations that are at risk for carrying α-globin gene rearrangements.

REFERENCES

1. Higgs DR, Vickers MA, Wilkie AO, Pretorius IM, Jarman AP, Weatherall DJ: A review of the molecular genetics of the human α-globin gene cluster. Blood 73:1081–104, 1989.
2. Higgs DR: α-Thalassaemia. Baillieres Clin Haematol 6:117–150, 1993.
3. Melis MA, Pirastu M, Galanello R, Furbetta M, Tuveri T, Cao A: Phenotypic effect of heterozygous α and β^0-thalassemia interaction. Blood 62:226–229, 1983.
4. Sampietro M, Cazzola M, Cappellini MD, Fiorelli G: The triplicated alpha-gene locus and heterozygous beta thalassaemia: A case of thalassaemia intermedia. Br J Haematol 55:709–710, 1983.
5. Thein SL, Al HI, Hoffbrand AV: Thalassaemia intermedia: A new molecular basis. Br J Haematol 56:333–337, 1984.
6. Galanello R, Paglietti E, Melis MA, Crobu MG, Addis M, Moi P, Cao A: Interaction of heterozygous β^0-thalassemia with single functional α-globin gene. Am J Hematol 29:63–66, 1988.
7. Oron V, Filon D, Oppenheim A, Rund D: Severe thalassemia intermedia caused by interaction of homozygosity for α-globin gene triplication with heterozygosity for β^0-thalassemia. Br J Haematol 86: 377–379, 1994.
8. Weatherall DJ, Clegg JB: The Thalassemia Syndromes, Ed 3. Oxford: Blackwell Scientific Publications, 1981.
9. Wainscoat J, Kanavis E, Wood W, Letsky E, Huehns E, Marsh G, Higgs D, Clegg J, Weatherall D: Thalassemia intermedia in Cyprus: The interaction of alpha and beta thalassemia. Br J Haematol 53:411–416, 1983.
10. Wainscoat JS, Old JM, Weatherall DJ: The molecular basis for the diversity of beta-thalassemia in Cypriots. Lancet ii:1235–1237, 1983.
11. Lauer J, Shen CK, Maniatis T: The chromosomal arrangement of human alpha-like globin genes: Sequence homology and alpha-globin gene deletions. Cell 20:119–130, 1980.
12. Goossens M, Kan YW: DNA analysis in the diagnosis of hemoglobin disorders. Methods Enzymol 76:805–817, 1981.
13. Bowden DK, Vickers MA, Higgs DR: A PCR-based strategy to detect the common severe determinants of alpha thalassaemia. Br J Haematol 81:104–108, 1992.
14. Dode C, Krishnamoorthy R, Lamb J, Rochette J: Rapid analysis of $-\alpha^{3.7}$ thalassaemia and $\alpha\alpha\alpha^{anti3.7}$ triplication by enzymatic amplification analysis. Br J Haematol 83:105–111, 1993.
15. Baysal E, Huisman THJ: Detection of common deletional α thalassemia-2 determinants by PCR. Am J Hematol 46:208–213, 1994.
16. Bowie LJ, Reddy PL, Nagabhushan M, Sevigny P: Detection of α-thalassemia by multiplex polymerase chain reaction. Clin Chem 40: 2260–2266, 1994.
17. Smetanina NS, Huisman THJ: Detection of α-thalassemia-2 (-3.7 kb) and its corresponding triplication ααα (anti-3.7 kb) by PCR: An improved technical change. Am J Hematol 53:202–203, 1996.
18. Kattamis AC, Camaschella C, Sivera P, Surrey S, Fortina P: Human α-thalassemia syndromes: Detection of molecular defects. Am J Hematol 53:81–91, 1996.
19. Nicholls RD, Fischel GN, Higgs DR: Recombination at the human alpha-globin gene cluster: Sequence features and topological constraints. Cell 49:369–378, 1987.
20. Chang JG, Liu TC, Chiou SS, Chen JT, Chen TP, Lin CP: Rapid detection of $-\alpha^{4.2}$ deletion of α-thalassemia-2 by polymerase chain reaction. Ann Hematol 69:205–209, 1994.